Institutionen för husdjursgenetik

Linkage mapping of phenotypic markers and marker identification in *MHC* in a cross between red junglefowl and Obese strain chicken

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Examensarbete 291

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Agrovoc: Chicken, feather colour, mapping, MHC, molecular marker

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1. Abstract

The aim of this project was to map the phenotypic traits plumage colour, feather pattern and shank colour and to identify an informative marker in exon 2 of the *MHC* class I locus in a cross between the Obese strain chicken and the red junglefowl. The three generation pedigree used was set up for study of spontaneous autoimmune thyroiditis (SAT) and generated through a reciprocal cross between the two lines. Obese strain chicken develops SAT and is a model for human Hashimoto's thyroiditis.

Individuals in the cross were scored for the *Dominant white* (*I*)/*PMEL17*, *Yellow legs* (*W*), *Inhibition of dermal melanin* (*Id*), *Extension* (*E*)/*MC1R*, *Sex-linked barring* (*B*) and *Stippling* loci. The *W* locus mapped to chromosome 24, *I* locus mapped to linkage group E22C19W28 and *Id* locus and *B* locus both mapped to chromosome Z, all as expected compared to prior mapping. The *E* and *Stippling* loci could not be mapped.

The length of the average linkage map including both sexes is 2358.85 cM with an average marker spacing of 6.63 cM and the male map is 7.3% longer than the female map. The map will later on be used for mapping of Quantitative Trait Loci.

Six SNPs were found in the start and end sequences of chromosome 16 but were not further analysed. One SNP was found in *MHC* but turned out not to be polymorphic in the cross when analysed with pyrosequencing. Microsatellite marker *LEI0258* was found to be informative in the cross.

2. Aim

The aim of this project was to perform a careful characterisation of phenotypic traits including plumage and shank colour and the secondary feather patterns stippling and barring and map these traits using a cross between the Obese strain chicken and the red junglefowl. Part of the project was also to identify an informative marker in chicken major histocompatibility complex (*MHC*) and genotype the entire pedigree for the marker with pyrosequencing. The mapping of colour and pattern is part of a continuous effort to map genes and markers in chicken and the marker in *MHC* is much needed in the research concerning spontaneous autoimmune thyroiditis in chicken.

3. Introduction

3.1. Chicken genetics

The chicken (*Gallus gallus*) genome consists of about 20,000 genes dispersed over 39 chromosome pairs out of which 38 pairs are autosomes and one pair is sex chromosomes (Wick *et al*. 2006). Eight pairs of the autosomes are large and cytogenetically distinct macrochromosomes and 30 pairs are small and cytogenetically indistinguishable microchromosomes. The microchromosomes comprise about 30% of the genome and have a higher gene density than the macrochromosomes (Smith *et al*. 2000). The chicken sex chromosomes are denoted Z and W and the female is heterogametic carrying Z and W while the male is homogametic carrying two Z chromosomes. The size of the chicken genome is approximately 1050 million base pairs (Mb), which is about 30% of the human genome. However, the genetic length of the chicken genome is of equal size to the human genome, about 4000 centiMorgan (cM) (Olofsson & Bernardi 1983, Wick *et al*. 2006).

The size difference is primarily due to the fact that the chicken genome contains fewer repetitive sequences compared to the human genome (Burt & Pourquie 2003, Wick *et al*. 2006). The explanation for the similar genetic length between the chicken and human genomes is that the recombination rate in chicken is higher than in human, 2.8 cM/Mb for macrochromosomes and 6.4 cM/Mb for microchromosomes compared to about 1 cM/Mb for humans. Translated this means that for chicken, 1 cM corresponds to about 300 kb while 1 cM in human corresponds to about 1000 kb. A high quality assembly of the chicken genome was published in 2004 (and updated in 2006) covering the major part of the chicken genome. A few areas are still poorly covered including the sex chromosomes and the *MHC* region on chromosome 16 (International Chicken Genome Sequencing Consortium 2004). However, the chicken *MHC* region was carefully studied and partly sequenced by Kaufman *et al*. (1999).

3.2. Pigmentation

There are two types of pigment in chicken, melanins and carotenoids. The melanins give colour to for example feathers, shanks and dark skin pigment while the carotenoids, especially xanthophyll, gives yellow colour to skin. Melanin is produced by melanocytes, which migrate to their location during embryogenesis (Smyth 1990). The melanins can be divided into eumelanin and pheomelanin and both are produced by the enzyme tyrosinase. The type of melanin produced depends on the tyrosinase activity level (Hearing & Tsukamoto 1991). Eumelanin (black melanin) gives black/brown pigmentation and pheomelanin gives yellow/red pigmentation (Smyth 1990).

3.2.1. Eumelanin

Eumelanin is distributed in primary and secondary patterns. Primary patterns are the local areas of pigment in the plumage of the bird whereas secondary patterns refer to distribution of pigment within individual feathers (Smyth 1990). There are a number of secondary patterns, but the focus of this project is on two specific patterns, barring and stippling, see Figure 1 and 2 for examples.

Stippling is inherited as an autosomal trait whereas *Barring* can appear both through autosomal inheritance and as a sex-linked trait (*B*). In this project, the *Sex-linked barring* is the one of interest. *Sex-linked barring* exerts a dosage effect, which means that homozygous individuals have wider bars than heterozygous individuals (Smyth 1990).
Responsible for the relative distribution of eumelanin and pheomelanin is the polyallelic *Extension* (*E*) locus. The two alleles of interest for this project are *Extended black* (*E*) and *wild-type* (e^+). The *E* allele enhances eumelanin synthesis whereas e^+ gives normal eumelanin synthesis and *E* is almost completely dominant over e^+ (Kerje *et al*. 2003b, Smyth 1990), however, the degree of dominance is influenced by genes able to enhance or inhibit eumelanin expression (Carefoot 1981). It has been established that the *Extended black* (*E*) locus is equivalent to the melanocortin 1-receptor gene (*MC1R*) (Kerje *et al*. 2003b). MC1R is a G-protein-coupled seven-transmembrane receptor expressed on melanocytes (Cone *et* al. 1996, Lu *et* al. 1994, Ollmann *et* al. 1998). The most likely causative mutation for the *Extended black* allele in *MC1R* is a E92K substitution leading to a constitutively active receptor (Kerje *et al*. 2003b).

Figure 1 Individuals having barred and stippled feather patterns. The top shows a barred female (left) and a barred male (right) and the bottom shows a stippled female (left) and a stippled male (right). In the male, stippling is displayed mainly in the tail feathers.

Figure 2 Examples of stippled (left) and barred (right) feathers.

Eumelanin synthesis is activated when α-melanocyte stimulating hormone (α-MSH) binds to MC1R whereas pheomelanin synthesis is activated when the antagonist from the a*gouti* allele binds to MC1R. *E*/*MC1R* has been assigned to chromosome 11 (Healy *et al*. 2001, Kerje *et al*. 2003b).

3.2.2. Pheomelanin

Pheomelanin is present in various shades of red wherever eumelanin is not present in wild-type plumage (Smyth 1990). One of the pheomelanin inhibitors is the sex-linked s*ilver* (*S*) mutation of the solute carrier family 24, member 2 (*SLC45A2*) (previously known as membrane-associated transporter protein (MATP)) (Gunnarsson *et al*. 2007, Smyth 1990). *Silver* is dominant to the g*old* (s^+) allele, which does not inhibit pheomelanin (Smyth 1990).

3.2.3. White plumage

White plumage, i.e. non-pigmented plumage, is caused by alleles at the *Dominant white* (*I*) and *recessive white* (*C*) loci in concert with a few other genes. The *Dominant white* (*I*) locus is equivalent to gene *PMEL17* (Kerje *et al*. 2004). *PMEL17* encodes the melanocyte-specific integral membrane protein PMEL17 which is of vital importance in the development of eumelanosomes (Berson *et al*. 2003, Du *et al*. 2003). The *Dominant white* (*I*) allele is associated with a mutation in *PMEL17*, namely a 9-bp insertion in exon 10, causing a three amino acid insertion in the PMEL17 transmembrane region. This insertion disrupts formation of normal eumelanosomes and the *I* allele is therefore a melanin, particularly eumelanin, inhibitor (Kerje *et al*. 2004). *I* removes black pigment from feathers and is incompletely dominant to the recessive wild-type allele i^+, which does not inhibit melanin. *I* is fairly ineffective in inhibiting pheomelanin and needs to be accompanied by for example the *E* and *S* alleles to be effective (Kerje *et al*. 2004, Smyth 1990). *Recessive white* (*c*) is a mutation in the *tyrosinase* gene (*C* locus) leading to a dysfunctional enzyme unable to produce pigment (Brumbaugh *et al*. 1983, Oetting *et al*. 1985).

3.2.4. Shank colour

Chicken shanks can appear in different colours, for example white, yellow, black, green and purple/blue. The shank colour is controlled by genes affecting different layers of the skin and the visible colour comes from the combined effect of eumelanin and xanthophyll in dermis and epidermis. There are mainly three loci controlling shank pigmentation, *Inhibition of dermal melanin* (*Id*), *Extension* (*E*), and *Yellow legs* (*W*), while others act as modifiers of shank colour (Smyth 1990). Dermal melanin pigmentation is controlled by the *Id* locus which is located on the Z chromosome, i.e. *Id* is sex-linked (Dunn 1925 and Punnett 1923 in Smyth 1990). The *Id* allele inhibits dermal melanin and is incompletely dominant to the wild-type id^+ allele, which does not inhibit dermal melanin (Smyth 1990). Epidermal melanin pigmentation in shanks is controlled by the alleles at the *E* locus (which also have some effect in dermis). In the absence of eumelanin inhibitors the shanks are black and *E*/*E* birds have pigmentation even when *Id* is present. The *W* locus

is responsible for xanthophyll (yellow pigment) deposition in the skin. The alleles at this locus are *white* (W^+) and *yellow* (*w*) and determine whether xanthophyll shall be deposited in the skin and shanks or not. W^+ prevents xanthophyll from entering the skin and by that also the shanks, hence resulting in white shanks whereas *w* allows deposition of xanthophyll leading to yellow skin and shanks (Smyth 1990). W^+ and *w* interacts with dermal melanization to create green or purple/blue shanks (Smyth 1990). A gene demonstrating complete linkage with the *W* locus has been identified (Jonas Eriksson personal communication). The modulatory genes affecting shank colour are *Sex-linked barring* (*B*), *Dominant white* (*I*) and *recessive white* (*c*). The *B* allele is a potent inhibitor of id^+ while *I* and *c* only dilutes dermal shank pigment (Knox 1935, Smyth 1990).

3.3. Genetic linkage

During meiosis, following replication, homologous chromosomes remain attached to each other so that there are four chromosome copies. This structure makes it possible for the arms of the chromosomes to overlay, break and exchange DNA segments. This rearrangement of the genome is called recombination or crossing-over (Figure 3) (Brown 2002).

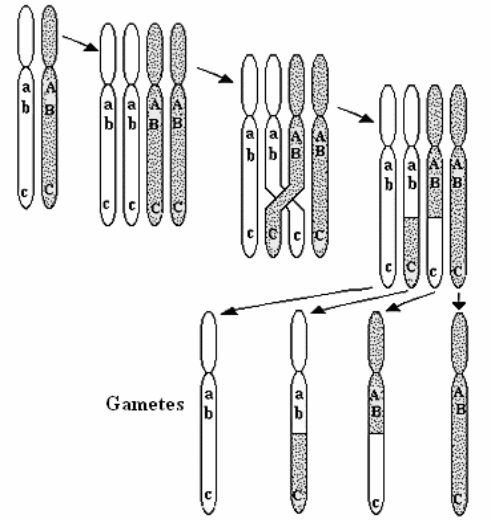

Figure 3 Recombination during meiosis.
Adapted from http://www.evolutionpages.com/homo_pan_divergence.htm

Recombination is a random event which can happen anywhere along the chromosome. Therefore genes located far apart will be separated more frequently than genes that are close to each other. Complete linkage indicates that the genes are in close proximity/right next to each other and that they are always inherited together in the offspring. Partial linkage is the result of recombination (Brown 2002). The recombination frequency between two markers/genes is directly proportional to the relative distance between them (and their relative map positions). A recombination frequency of 1% corresponds to a distance of 1 centiMorgan (cM) (Brown 2002, Wick *et al*. 2006). The map distance does not always indicate the actual physical distance between the genes on the chromosome because of the presence of recombination hotspots (high recombination) and coldspots (low recombination) (Brown 2002, Koren *et al*. 2002).

3.3.1. Linkage mapping

Linkage mapping is a way of mapping traits, assigning markers or genes to a relative position in the genome, and locating chromosomal regions harbouring one or several causative genes for the genotype studied (Brown 2002, Wick *et al*. 2006). A linkage mapping experiment starts with the creation of a pedigree of at least three generations in which the locus/loci controlling the studied phenotype/trait segregates and the genotypes of the parentals are known (Brown 2002). All individuals in the F_1 generation created from the crossing of the two parental strains (carrying different alleles at the trait locus/loci) will be heterozygous at the trait locus/loci. The following generation, the F_2 generation, can be created either by intercrossing animals from the F_1 generation or by backcrossing individuals from the F_1 generation with the parental line displaying the trait depending on the complexity of the trait. The next step is to collect phenotypic data and DNA samples from the entire pedigree and use it in a genome scan with genetic markers. After the genome scan, a statistical analysis is carried out to find the markers cosegregating with the trait locus/loci. If a marker is positioned close to the trait loci on the chromosome it will show limited or no recombination between itself and the trait loci and this will indicate where the trait is located. When the chromosomal region harbouring the causative gene has been located the actual causative gene must be found and confirmed (Wick *et al*. 2006).

3.3.2. Genetic Markers

A detailed genetic map consists of the DNA markers restriction fragment length polymorphisms (RFLPs), simple sequence length polymorphisms (SSLPs), minisatellites and microsatellites, and single nucleotide polymorphisms (SNPs) (Brown 2002). Microsatellites are popular due to their high degree of polymorphism, their size and their even spread throughout the genome, however for this study SNPs has been used. SNPs are positions in the genome where, between individuals, the nucleotides present differ. There could theoretically be four alleles at each SNP, however in reality there are often just two. Because of this, the SNPs are limited as DNA markers (Brown 2002). The advantages with SNPs, and why they are the most popular markers today, are their rich number in the genome, 2.8 million (International Chicken Polymorphism Map Consortium 2004), and the methods available for typing (Brown 2002, Siegel *et al*. 2006). One typing method is pyrosequencing by which shorter DNA segments can be sequenced. One nucleotide (dNTP) at the time is added to the solution containing the DNA strand to be sequenced following a pre-determined schedule creating a growing polynucleotide strand and if the nucleotide hybridizes with the DNA sequence a pyrophosphate molecule is released and converted by the enzyme sulfurylase to a flash of chemiluminescence. Thereby you can follow the nucleotide incorporation and get the sequence. The advantage with pyrosequencing is that no gel electrophoresis isolating step is needed as the PCR products can be directly analysed (Ronaghi *et al*. 1998). Any left over nucleotides are degraded by a nucleotidase before the next one is added (Brown 2002).

3.4. The red junglefowl and Obese strain (OS) chickens

The cross used in this project is between red junglefowl and Obese strain (OS) chicken. The red junglefowl is considered to be the wild ancestor of the domestic fowl and by that carrying the wild-type alleles (Fumihito *et al*. 1994, Smyth 1990). Compared to the domestic White Leghorn (OS), the red junglefowl differ in a number of ways. The red junglefowl weigh less, reach sexual maturity at different time, lay fewer eggs, have a different behaviour and have coloured plumage (Schütz *et al*. 2002). Furthermore, the red junglefowl female lacks comb (Kaul *et al*. 2004). The cross used in this project was originally set up for studies of the spontaneous autoimmune thyroiditis (SAT) manifested in chickens from the Obese strain. The OS chickens are a close-bred strain that develops SAT about one week after hatching and are expected to be fixed or to be close to fixation for the alleles causing the disease. SAT is very similar to the human disease Hashimoto's thyroiditis and OS chicken is therefore a good disease model (Kühr *et al*. 1994, Neu *et al*. 1986, Wick *et al*. 2006). SAT is manifested as hypothyroidism, giving the OS chickens their distinctive phenotype. The phenotypic signs are small body size, large amounts of subcutaneous fat, which gives relatively high body weight (obese), long silky feathers, small combs, low fertility and poor hatchability (Neu *et al*. 1986, Wick *et al*. 2006). Figure 4 shows OS chickens and Figure 5 shows red junglefowl chickens.

Figure 4 Examples of OS animals suffering from SAT, female (left) and male (right). The bottom photo shows the characteristic long silky feathers associated with SAT.

Figure 5 Red junglefowl. Male (left) (http://www6.plala.or.jp/ttaguphoto/photo/red-unglefowl-2.jpg) and female (right)
(http://orientalbirdimages.org/images/data/7100_red_junglefowl_f_pp.jpg)

There are certain *MHC* haplotypes (*B* locus) associated with SAT. *B13* and *B15* are associated with severe disease and *B5* with mild disease. However, the *MHC* is believed to have only a modulatory and fine tuning role rather than being a prerequisite for SAT (Hala 1988).

3.5. MHC

The major histocompatibility complex (*MHC*) of domestic chicken, *Gallus gallus domesticus*, consists of two genetically independent (unlinked) clusters of *MHC* genes located on chromosome 16 separated by the nucleolar organizer region (NOR) (Figure 6). The gene clusters are the classical *B* system and the *restriction fragment pattern-Y* (*Rfp-Y*) system (Afanassieff *et al*. 2001, Miller *et al*. 1996). Both clusters contain class I and II *MHC* (*B*) genes and the gene products from the class I and II genes are of large importance in the immune system where they bind and present foreign peptides to T-cells (Afanassieff *et al*. 2001, Wittzell *et al*. 1999).

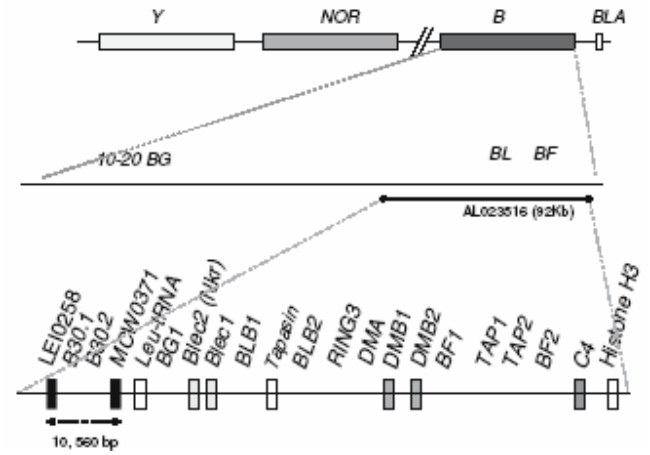

Figure 6 *MHC* gene arrangement. Adapted from Fulton *et al*. 2006.

The B system is made up of closely linked polymorphic regions: *BF* (class I), *BL* (class IIβ) and *BG* (Ig-superfamily genes). The *BF*/*BL* region consists of 19 genes and is 92 kilobases large (Ruby *et al*. 2005). There is a strong linkage disequilibrium between the

alleles at *BF*, *BL* and *BG* loci creating haplotypes and these haplotypes are considered to have large influence on an individual's ability to mount an efficient immune response. Therefore it is appropriate to consider the entire haplotypes instead of individual loci (Fulton *et al*. 2006, Iglesias *et* al. 2002, Kaufman *et al*. 1999). The *MHC* is highly polymorphic in most mammals, for example human and mouse, as well as in chicken (Goldsby *et al*. 2003, Livant *et al*. 2004). The organisation of the chicken *MHC* differs from mammalian and is smaller and more compact (Miller *et al*. 2004). A part of class I peptide binding region (PBR) is encoded by exon 2. Exon 2 is therefore highly polymorphic and suitable for SNP search (Bonneaud *et* al. 2004, Shimizu *et al*. 2004, Thoraval *et al*. 2003). There are two known microsatellite markers in the chicken *MHC*, *MCW0371* and *LEI0258*. Both are located between the *BG* and the *BF* regions in the *MHC B* locus (Fulton *et al*. 2006). In other species, there have been indications that *MHC* class II is important for autoimmune disease (Martinez *et al*. 2007). To be able to test the relevance of *MHC* for SAT an informative marker in *MHC* is needed. Since there is linkage disequilibrium (Fulton *et al*. 2006, Kaufman *et al*. 1999) in the entire *B* system of *MHC*, a marker located anywhere in the *B* system will be sufficient. If an informative marker is found in *MHC* or on another location on chromosome 16 it is possible to search for linkage to *MHC* and chromosome 16.

4. Materials and methods

4.1. Animal material and phenotyping

A three-generation pedigree was used in the study. A reciprocal cross was generated after mating four red junglefowls, two males and two females, with nine OS chickens, one male and eight females (Figure 7). 43 animals (eight males and 35 females) from the F_1 generation were intercrossed producing 759 F_2 animals, which were used for phenotypic classification. The classification was made from digital photos and collected feathers. The phenotypes classified were plumage colour, shank colour and the feather patterns stippling and barring.

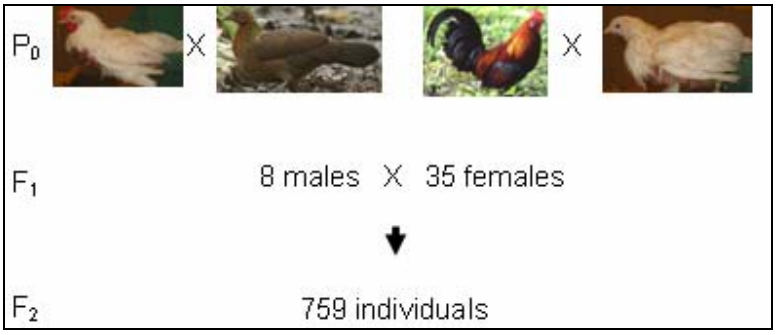

Figure 7 Illustration of how the reciprocal pedigree was created.

4.1.1. Shank colour

Phenotypic classification was made distinguishing between six different shank colours. In Table 1 the colours and the responsible alleles according to Smyth (1990) at loci *W*, *E* and *Id* are shown. The phenotype yellow spotted has no assigned alleles but was tested

also as yellow in the statistical analysis because of uncertainty about how to classify the individuals for the *W*, *E* and *Id* loci.

Table 1 Shank colour and alleles responsible (from Smyth 1990).

Pigment				
Carotenoid	**Dermal melanin**	**Epidermal melanin**	**Genotype**	**Phenotype**
W+	Id	e+	W+/W+ Id/Id e+/e+	White
*	id+	E	*/* id+/id+ E/E	Black
W+	id+	e+	W+/W+ id+/id+ e+/e+	Purple/Blue
w	Id	e+	w/w Id/Id e+/e+	Yellow
w	id+	e+	w/w id+/id+ e+/e+	Green

The shank colours were obtained at 28 weeks of age for correct classification, see Appendix A for photographic examples of the shank colours. For the individuals with black shanks the alleles at the *W* locus could not be classified due to that the sole colour could not be seen from the photos.

4.1.2. Plumage colour

Phenotypic classification was made distinguishing between white and coloured plumage (classification of *I* locus). Animals with white plumage were classified as having at least one *I* allele and animals with coloured plumage were classified as being homozygous for the id^+ allele, see Appendix B for photographic examples of plumage colours.

4.1.3. Feather patterns

Phenotypic classification of the secondary patterns stippling and barring was made. The criteria for stippling were set as various amounts of black spots anywhere on the body on white background plumage. The criteria for barring were set as barred feathers anywhere on the body of the animal on white or coloured background plumage. The classifications for stippling were done both considering the *Stippling* gene being dominant and recessive. When dominant, stippled animals were classified as having at least one S*tippling* allele whereas animals without stippling were classified as being homozygous for the *wild-type* allele at the *Stippling* locus. When recessive, stippled animals were classified to be homozygous for the *Stippling* allele whereas animals without stippling were classified as having at least one *wild-type* allele at the S*tippling* locus. Barred animals were classified as having at least one *B* allele whereas non-barred animals were considered to be homozygous for the *wild-type* allele at the *B* locus.

4.1.4. Genotyping and genetic map

Segregation analysis of shank colour, plumage colour and feather pattern was performed comparing observed results with expected Mendelian segregation ratios using chi-square tests. A twopoint linkage analysis was then performed using the CRI-MAP (version 2.4) software (Green *et al*. 1990) testing the phenotype markers for linkage to the 356 SNP markers already typed in the pedigree using the Illumina system (Sahlqvist in preparation). A LOD score value of 3 was set as a criterion for significant linkage. To

position the phenotype markers on the linkage map and find out the order of markers, the functions BUILD, FIXED, FLIPS and CHROMPIC were used in the CRI-MAP software.

4.2. Sequence analysis and detection of single nucleotide polymorphism (SNP)

Genomic DNA was isolated by standard methods (DNA Isolation Kit, AGOWA, Berlin, Germany) and used for PCR amplification of the start and end sequences, *MHC* and *LEI0258* on chromosome 16 for all parentals.

4.2.1. Chromosome 16 start and end

Primer pairs were designed at the chromosome ends, distal parts of the p (start) and q (end) arms, from the chicken genome sequence (http://genome.ucsc.edu) with the software Primer3 (primer3_www.cgiv0.2). A 1081 bp segment in the start of chromosome 16 and a 1018 bp segment in the end of chromosome 16 were amplified. The primers Chr16_start_fwd (5'-GACATACGAGCTGAGTAGGG-3') and Chr16_start_rev (5'-GGACACAGTACAGGGTTAGG-3') amplified the 1081 bp segment in the start of the chromosome and the primers Chr16_end_fwd (5'-TGTGGTGACAAACCTTACAA-3') and Chr16_end_rev (5'-CTGTGTGGTGAGAAGTGTTG-3') amplified the 1018 bp segment in the end of the chromosome. A total volume of 10 µl was used and the PCR reaction contained about 20 ng genomic DNA, 1x PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$ (Applied Biosystems), 200 µM deoxynucleotide triphosphate (dNTP), 0.5 U AmpliTaq Gold™ DNA polymerase (Applied Biosystems) and 2 pmol of each primer. The PCR started with 4 min at 94°C followed by 35 cycles with 30 sec at 94°C, 30 sec at 55°C, 1 min 20 sec at 72°C and ending with 5 min incubation at 72°C. The PCR products were isolated on an agarose gel and purified with E.Z.N.A. Gel Extraction Kit 200 (Omega Bio-tek, Doraville, GA, USA) and sequenced with DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (Amersham Biosciences, Uppsala, Sweden). The same primers used in the PCR were used for the sequencing. Sequences were analysed using the Sequencher™ 3.1.1. software (Gene Codes Corporation, Inc, Ann Arbor, MI, USA) in order to correct sequencing errors and identify possible polymorphisms. The fragments were not analysed further using pyrosequencing because of limited time.

4.2.2. *MHC*

The primers MHC_BF1_fwd (5'-TGCCCCGCACCGAGTGGAT-3') and MHC_BF1_rev (5'-CCGGTCTGGTTGTATCGTTC-3') amplified a 133 bp segment in exon 2 of *MHC* class I locus *BF1* located on chromosome 16 (Bed'hom *et al*. 2006). A total volume of 20 µl was used and the PCR reaction contained about 40 ng genomic DNA, 1x PCR buffer II (Applied Biosystems), 2.5 mM $MgCl_2$ (Applied Biosystems), 200 µM deoxynucleotide triphosphate (dNTP), 1 U AmpliTaq Gold™ DNA polymerase (Applied Biosystems) and 4 pmol of each primer. The PCR started with 4 min at 94°C followed by 40 cycles with 30 sec at 94°C, 30 sec at 55°C, 30 sec at 72°C and ending with 5 min incubation at 72°C. The PCR products were isolated on an agarose gel and

purified with E.Z.N.A. Gel Extraction Kit 200 (Omega Bio-tek) and sequenced with DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE DNA Analysis Systems (Amersham Biosciences). The same primers used for the PCR was used for the sequencing. Sequences were analysed using the Sequencher^{TM} 3.1.1. software (Gene Codes Corporation) in order to correct sequencing errors and identify possible polymorphisms. The SNP found was analysed for all animals by pyrosequencing. The primers used for pyrosequencing PCR were MHC_BF1_fwd and chMHC_bio_rev (5'-Biotin-CCGGTCTGGTTGTATCGTTC-3') and the PCR program was the same as for the start and end sequences mentioned earlier. The pyrosequencing reactions were run with primer chMHC_seq (5'-CGGACCAGCAGTACTGG-3') (Bed'hom *et al*. 2006).

4.2.3 Microsatellite *LEI0258*

The PCR primers forward (5'-HEX-CACGCAGCAGAACTTGGTAAGG-3') and reverse (5'-AGCTGTGCTCAGTCCTCAGTGC-3') (McConnell *et al*. 1999) were used to amplify the LEI0258 microsatellite marker. A total volume of 10 µl was used and the PCR reaction contained about 50 ng genomic DNA, 1x PCR Buffer II (Applied Biosystems), 1.5 mM $MgCl_2$ (Applied Biosystems), 200 µM deoxynucleotide triphosphate (dNTP), 0.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems) and 5 pmol of each primer. The PCR started with 1 min at 94°C followed by 40 cycles with 45 sec at 92°C, 45 sec at 57°C, 45 sec at 72°C and ending with 1.5 hour incubation at 72°C. The PCR product were denatured 1 min before electrophoresis in 4% polyacrylamide gel in the MegaBACE capillary instrument (Amersham Biosciences) and results were analysed with GeneticProfiler version 2.2 (Amersham Biosciences).

5. Results

5.1. Mapping

The *W* locus was mapped to chromosome 24 with a recombination fraction between *W* and marker *RS15221797* of 0.16 and a LOD score of 5.5. Marker *RS15221797* is known to be on chromosome 24 and this position of *W* is in correlation with prior mapping. The *I* locus was mapped to linkage group E22C19W28 between marker *RS15989709*, recombination fraction 0.09 and LOD score 22.57, and *RBL3889*, recombination fraction 0.06 and LOD score 49.16. This position of *I* is in correlation with prior mapping (Ruyter-Spira *et al*. 1996, Schmid *et al*. 2000). Loci *Id* and *B* mapped to chromosome Z as expected. They mapped to the end region of chromosome Z with a recombination fraction between them of 0.09 and a LOD score of 53.16. Between *Id* and marker *RS16121860* the recombination fraction is 0.25 and the LOD score 12.98. This mapping strengthens prior mapping of *Id* and *B*. *E* and S*tippling* could not be mapped with confidence. The recombination fractions and LOD scores displayed here comes from the twopoint analysis and might differ a bit from the recombination fractions in the linkage maps but that is in order. See Appendix C for the generated linkage maps over chromosomes 24, linkage group E22C19W28 and chromosome Z. For chromosome 24 and linkage group E22C19W28, both a sex-average and a sex-specific map were made but for chromosome Z only the sex-specific map was made. The Z chromosome map

represents recombination in males as females are heterogametic and can have no recombination between the sex chromosomes.

The average map length in this cross is 2358.85 cM with an average marker spacing of 6.63 cM. The female map is 2275.5 cM with an average marker spacing of 6.39 cM and the male map is 2442.2 cM with an average marker spacing of 6.86 cM. The male map is 7.3% longer than the female map.

The effect of the *I* allele on shank colour was calculated with a chi-square test and as expected, *I* affects only the most pigmented colours, black and purple (Table 2).

Table 2 The effect of *I* locus on shank colour.

I_LOC	I/-	i/i	tot	chi2 1 df	significance
BLACK	52	42	94	19,42	***
GREEN	12	9	21	3,57	
PURPLE	142	29	171	5,9	*
SPOTTED	3	1	4	0	
WHITE	210	65	275	0,27	
YELLOW	58	18	76	0,07	

The number of white animals compared to coloured animals gives the ratio 3:1.

5.2. SNPs on chromosome 16

In the start sequence of chromosome 16 three informative SNPs were detected. Two of them had individuals showing heterozygosity indicating that these SNPs are not totally informative. Markers are informative when the alleles between the two crossed strains differ. With informative markers, the inheritance can be monitored. In Table 3 the base-pair positions of the SNPs and the nucleotides/alleles present for OS and JF compared to the reference sequence in the start sequence are shown.

Table 3 SNPs in the start sequence.

Position	Ref. sequence	OS	JF	Heterozygosity
434	G	G	A	A/G
472	A	A	G	A/G
496	C	C	G	

In the end sequence of chromosome 16 three more informative SNPs were detected. None of these SNPs displayed any heterozygosity and seems to be totally informative. In Table 4 the base-pair positions of the SNPs and the nucleotides/alleles present for OS and JF compared to the reference sequence in the end sequence are shown. See Appendix D for illustrations of the SNPs found in the start and end sequences.

Table 4 SNPs in the end sequence.

Position	Ref. sequence	OS	JF
2960	C	C	T
2995	C	T	C
3097	G	A	G

In the exon 2 sequence, one SNP was detected. The base-pair position is 55 and the nucleotides are G for OS, T for JF and G for the reference sequence, see Appendix E for illustration of the SNP found in exon 2.

Pyrosequencing analysis resulted in the tested animals all being homozygous for G/G at the exon 2 SNP when expected to be G/T in OS and G/G in JF. Hence, there is no heterozygosity/sequence polymorphism in the sequenced region. See Appendix F for an example of the pyrogram.

5.3 Microsatellite LEI0258

The microsatellite marker *LEI0258* was tested for variation in the pedigree. It turned out to be informative and will be used as a marker in *MHC*. The OS individuals show fixation for one *LEI0258* allele (size 209 bp) as expected from a close to inbred line whereas JF show allelic variation, two alleles (size 197 bp and 352 bp) for *LEI0258* as expected from a wild strain.

6. Discussion

We were able to map the loci *I*, *W*, *Id* and *B* with significance. These results are in correlation with previous results and the obtained linkage mapping results can in the future be used to identify the genes responsible for *Id* and *B*. To be able to identify genes, a good map position is crucial to know where to look. However, in the most recent chicken genome assembly (September 2006) the sex chromosomes are still poorly covered and because of this, gene identification can be difficult in these areas as part of the sequence is missing.

There are earlier results indicating that *Id* and *B* might in fact be a single locus (Leif Andersson personal communication). We found recombination between the two loci indicating that this is not true (0.09 and a LOD score of 53.16 (twopoint)). However, they may not be located that far apart since there is more recombination in the ends of chromosomes where these loci are located.

We were not able to map the *E* and S*tippling* loci. A possible explanation concerning the *E* loci is that it might not be possible to map *E* from shank colour. If the shank phenotypes black and purple/blue are confused, the assigned *E* allele will be incorrect. Furthermore, the loci modulating shank colour, *I* and *B*, and gene interactions might cause uncertainty to the classification of *E*. We have shown that the *I* loci affects shank colour since the distribution of the *I* allele for the different shank colours significantly deviates from the classical Mendelian ratio of 1:2:1, which it would display if it had no effect on shank colour. Concerning the failure of mapping S*tippling*; the criteria we set up may have been incorrect but it is possible to treat S*tippling* as a trait and use it in a Quantitative Trait Loci analysis instead.

As a measurement of the *I* loci distribution for plumage colour, the ratio of white plumage compared to coloured plumage was calculated. The ratio is 3:1 which also is what to expect from the dominant nature of the *Dominant white* (*I*) *allele*.

In this cross the male map is 7.3% longer than the females map. This is the opposite from what has been observed in other crosses and species. Normally the female map tends to be longer than the male map, the pig female map is 40% longer than male

(Bidanel *et al*. 2001, Marklund *et al*. 1996), human female map is 70% longer than male (Morton 1991) and JF x WL female map 8% longer than male (Kerje *et al*. 2003a). However, there is a postulation by Haldane (1922) saying that the homogametic sex (females in mammals and males in birds) has a higher degree of recombination and thereby longer genetic map. Our result correlates with the postulation. There can be many explanations to why the map length is different between sexes and no one knows for sure. The longer female map in the JF x WL cross indicates a higher recombination frequency in females. One explanation for this can be gamete production. As males produce many more gametes than females and have a good chance of recombinants anyway, maybe females need to have a higher recombination frequency than males to create a balance between the sexes.

The JF x OS map created for our cross is shorter than previous maps which is a bit unexpected. We expected our JF x OS map to be longer than the JF x WL map described in Kerje *et al*. (2003a) since our map cover a larger part of the genome than the JF x WL map. We do not know the reason for this, however the indications are that there is less recombination in OS than in WL. Furthermore, our map is made up of only SNPs, no microsatellites as in Kerje *et al*. (2003a), and there might be a difference in the informativeness of the markers. To find the reason for the short map, a microsatellite can be run to see if the inheritance is correct.

The *MHC* SNP turned out not to be a polymorphism. An explanation to this can be that the sequences used only were sequenced in one direction. A marker is needed in *MHC* because it is highly desirable to study the role of *MHC* in spontaneous autoimmune thyroiditis. It has been postulated that the role of *MHC* is only modulatory but this needs to be explored further. What we want to know if the haplotypes associated with severe disease, *B13* and *B15*, and mild disease, *B5*, are present in the cross and if they are of importance to spontaneous autoimmune thyroiditis. The marker to be used in *MHC* is *LEI0258*.

7. Acknowledgements

I would like to thank my supervisor, Susanne Kerje for help and support, Anna-Stina Sahlqvist for photos and help, Ulla Gustafson for sequencing, Göran Andersson, my examiner, the whole Department of Animal Breeding and Genetics and my friend Katarina Davidsson for brightening my days.

8. Swedish summary

Målet med detta projekt var att i en korsning mellan röd djungelhöna och Obese strain höna kartlägga, med kopplingsanalys, de fenotypiska markörerna fjäderdräktsfärg, fjädermönster och benfärg samt att identifiera en informativ markör i major histocompatibility complex (*MHC*). Den röda djungelhönan anses vara anfadern till dagens domesticerade höna och Obese strain hönan utvecklar spontan autoimmun tyroidit och är modelldjur för den humana sjukdomen Hashimotos tyroidit. De aktuella lokusen involverade i de fenotypiska markörerna är *Dominant vit* (*I*)/*PMEL17*, *Gula ben* (*W*), *Inhibering av dermalt melanin* (*Id*), *Extension* (*E*)/*MC1R*, *Barring* (*B*) och S*tippling*. *W* lokus kartlades till kromosom 24, *I* lokus till kopplingsgrupp E22C19W28 och *Id* lokus

och *B* lokus båda till kromosom Z. Kartläggningen av *W*, *I*, *Id* och *B* stämmer överens med tidigare resultat men *E* lokus och S*tippling* kunde inte kartläggas. Det finns fler lokus som påverkar färg på fjädrar och ben. Dessa lokus tillsammans med geninteraktioner kan ha bidragit till att *E* inte kunde kartläggas. Den genomsnittliga kartlängden i denna korsning är 2358.85 cM med ett genomsnittligt markköravstånd av 6.63 cM och den hanliga kartan är 7.3% längre än den honliga. Sex enkel nukleotid variationer hittades i start- och slutsekvenserna av kromosom 16 men analyserades inte vidare.

För att kunna studera relationen mellan *MHC* och spontan autoimmun tyroidit så behövs det en eller flera markörer i *MHC*. Mikrosatellitmarkören *LEI0258* testades och visade sig vara informativ i korsningen. Fortsatta studier kommer att undersöka om tidigare nämnda *MHC* haplotyper är kopplade till svår (*B13* och *B15*) eller lindrig (*B5*) sjukdom.

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Appendix A, Shank colours

White (W)

Black (B)

Yellow (Y)

Purple (P)

Green (G)

Yellow spotted (S)

Appendix B, Examples of plumage colours

White plumage

Coloured plumage

Appendix C, Linkage maps

```
Sex_averaged map (recomb. frac., Kosambi cM):

  0   RS15221797                  0.0
                  0.21   22.8
  1   W_LOC                      22.8

* denotes recomb. frac. held fixed in this analysis

log10_like = 69.34




Sex-specific map (recomb. frac., Kosambi cM -- female, male ):

  0   RS15221797                  0.0                                0.0
                  0.21   22.8                  0.21   22.8
  1   W_LOC                      22.8                               22.8

* denotes recomb. frac. held fixed in this analysis

log10_like = 69.34
```

Chromosome 24 sex-average and sex-specific maps

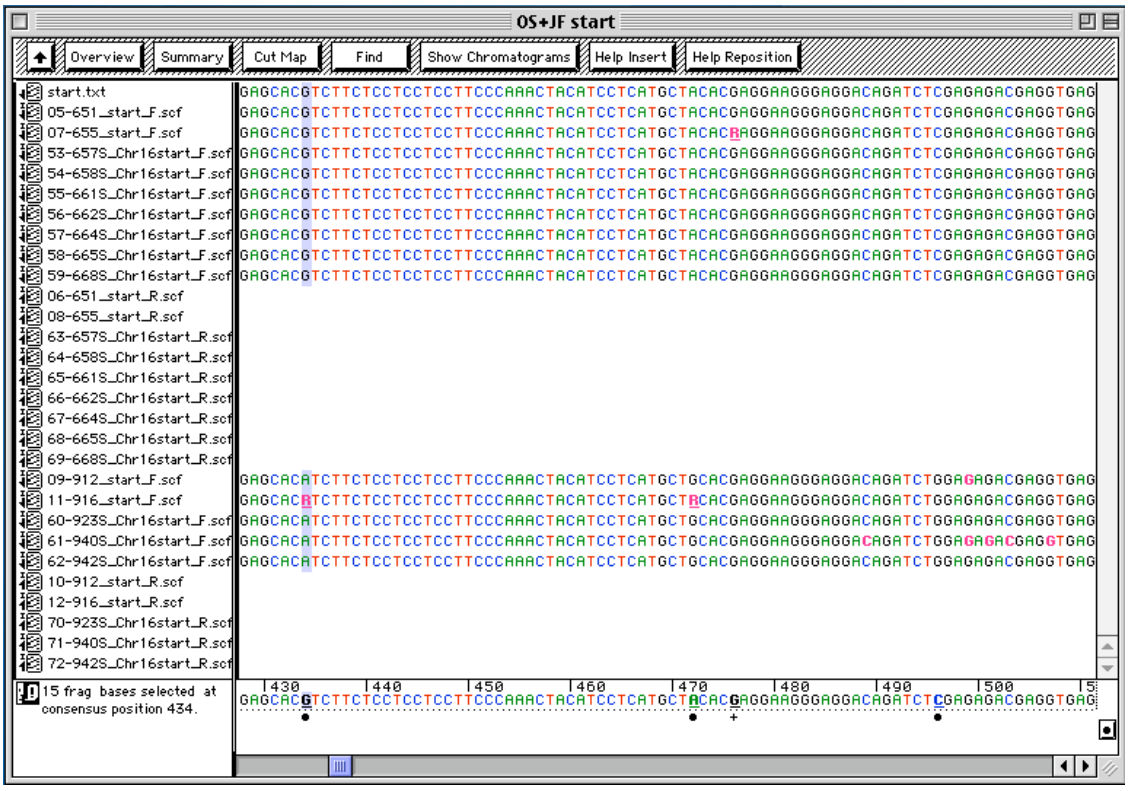
```
Sex_averaged map (recomb. frac., Kosambi cM):

  3   RS15989709                    0.0
                    0.11   11.4
  2   I_LOC                        11.4
                    0.06    6.5
  1   RBL3889                      17.9
                    0.12   12.7
  0   RBL530                       30.6

* denotes recomb. frac. held fixed in this analysis

log10_like = -235.20




Sex-specific map (recomb. frac., Kosambi cM -- female, male ):

  3   RS15989709                    0.0                                  0.0
                    0.09    8.9                   0.13   13.3
  2   I_LOC                         8.9                                 13.3
                    0.09    9.1                   0.04    4.1
  1   RBL3889                      18.0                                 17.3
                    0.14   13.9                   0.11   11.7
  0   RBL530                       31.8                                 29.0

* denotes recomb. frac. held fixed in this analysis

log10_like = -234.73
```

Linkage group E22C19W28 sex-average and sex-specific maps

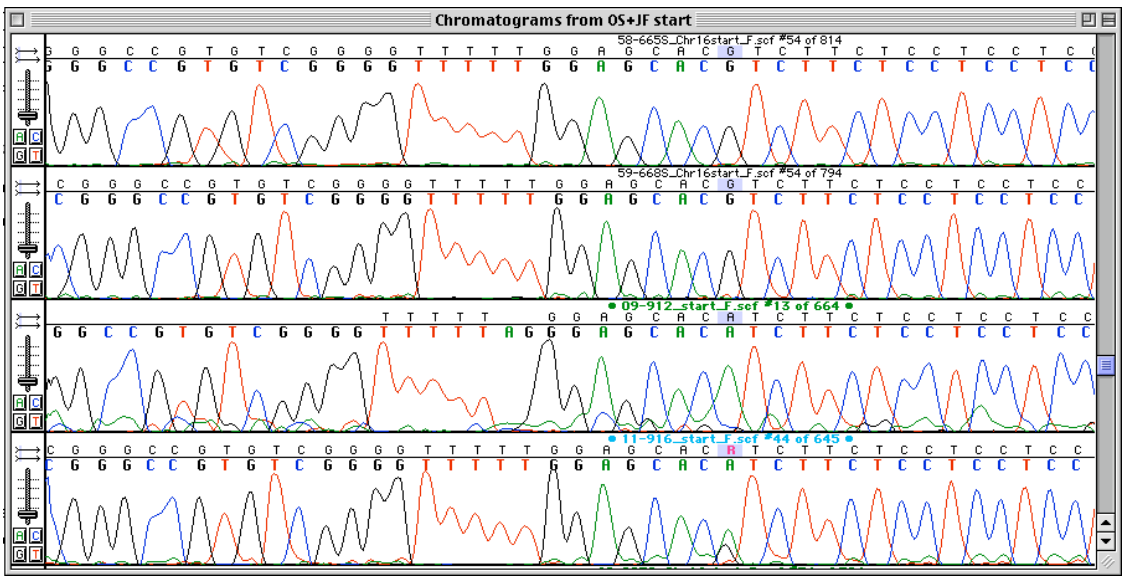
```
Sex-specific map (recomb. frac., Kosambi cM -- female, male ):

  0   RS16098196                0.0                          0.0
                    0.00    0.0                   0.32   38.6
  1   RS16103283                0.0                         38.6
                    0.00    0.0                   0.07    7.5
  2   RS16100280                0.0                         46.1
                    0.00    0.0                   0.15   15.7
  3   RS16105339                0.0                         61.8
                    0.00    0.0                   0.15   15.3
  4   RS16106682                0.0                         77.0
                    0.00    0.0                   0.02    1.9
  5   RS14762832                0.0                         78.9
                    0.00    0.0                   0.13   12.9
  6   RS16111451                0.0                         91.8
                    0.00    0.0                   0.16   16.9
  7   RS16112880                0.0                        108.8
                    0.00    0.0                   0.01    0.7
  8   RBL1380                   0.0                        109.5
                    0.00    0.0                   0.04    4.1
  9   RS16114675                0.0                        113.6
                    0.00    0.0                   0.12   11.7
 10   RS16116788                0.0                        125.3
                    0.00    0.0                   0.13   13.4
 11   RS16119736                0.0                        138.8
                    0.00    0.0                   0.11   11.4
 12   RS16121860                0.0                        150.2
                    0.00    0.0                   0.35   43.3
 14   ID_LOC                    0.0                        193.5
                    0.00    0.0                   0.15   15.2
 13   B_LOC                     0.0                        208.8

* denotes recomb. frac. held fixed in this analysis

log10_like = -1469.47
```

Chromosome Z sex specific map

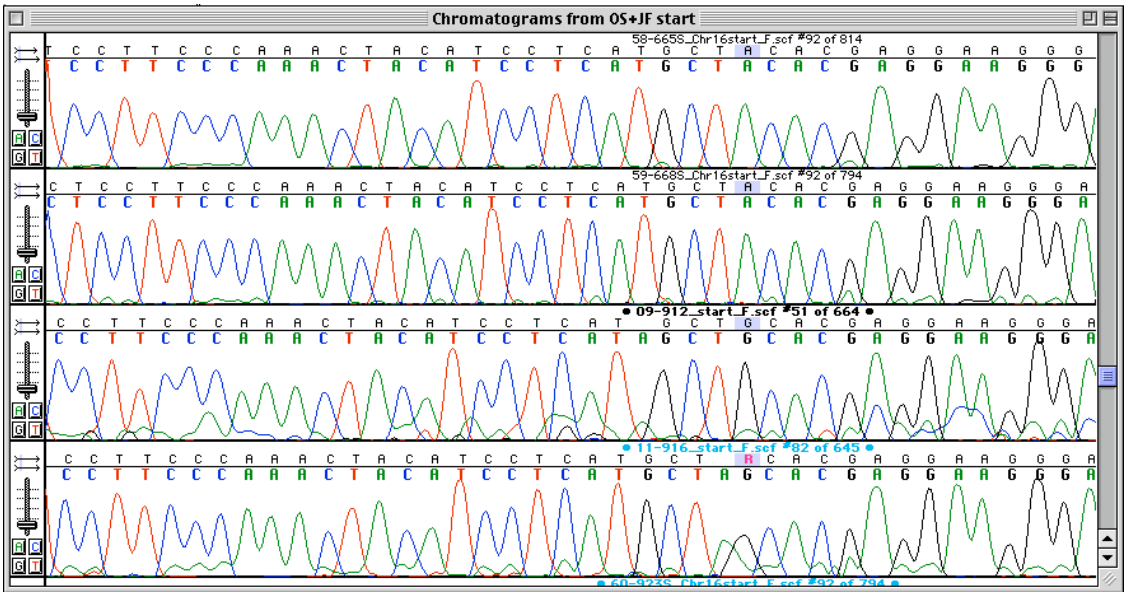
Appendix D, SNPs start and end



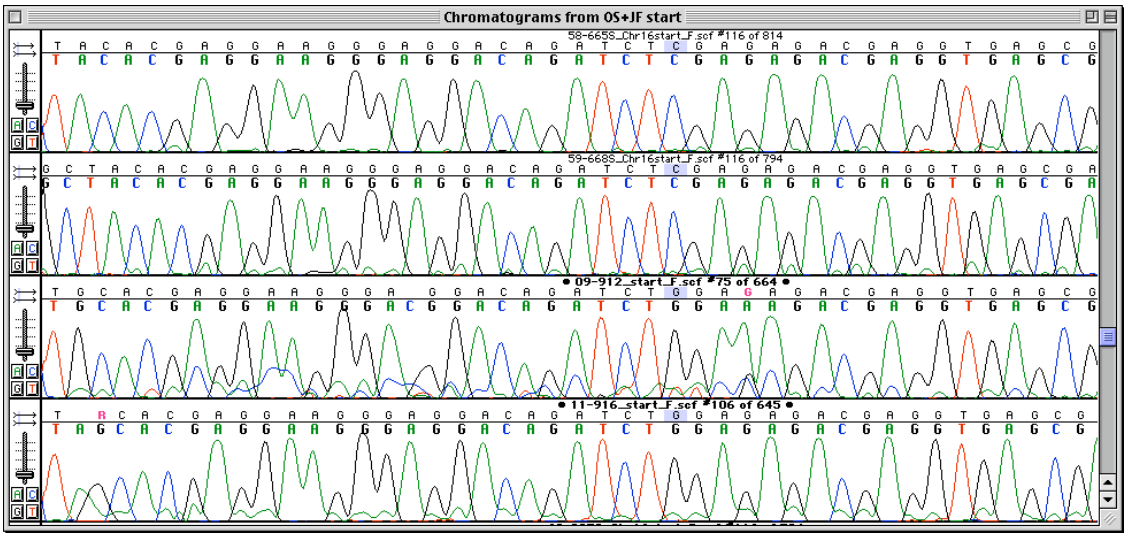
Informative SNPs found in the start sequence. In base-pair position 434, the OS chickens have G and JF A; in base-pair position 472, the OS chickens have A and JF G and in base-pair position 496, the OS chickens have C and JF G.



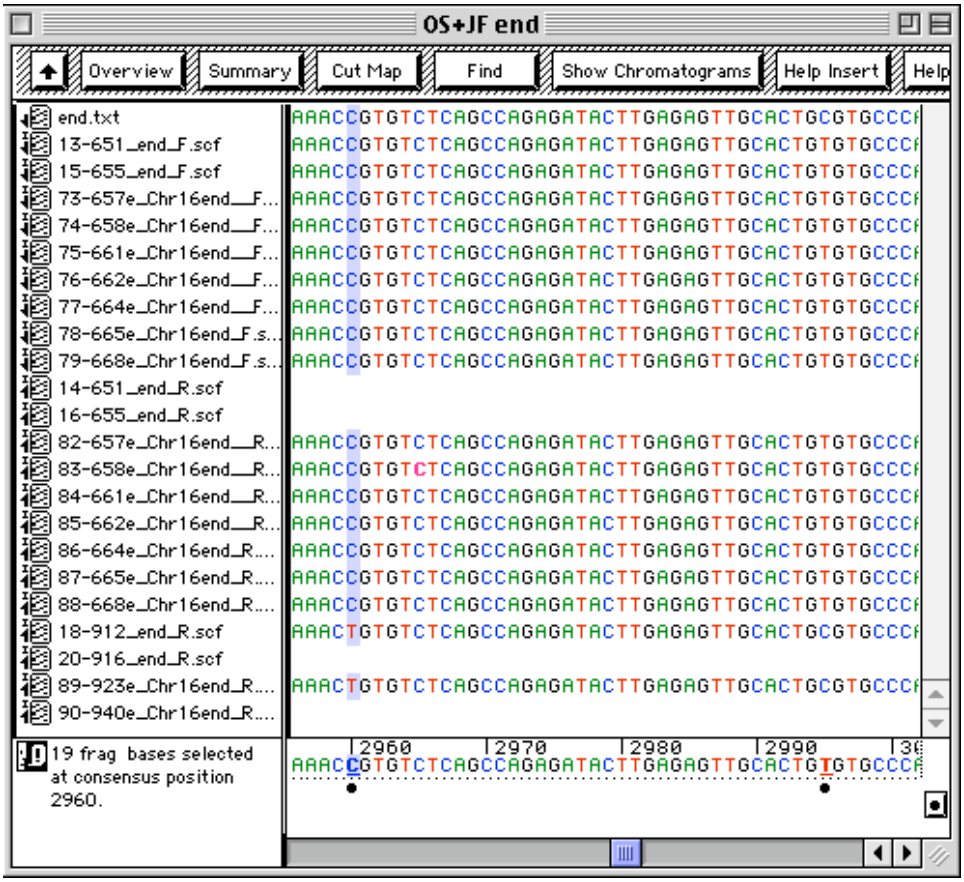
Base-pair position 434 in the start sequence.



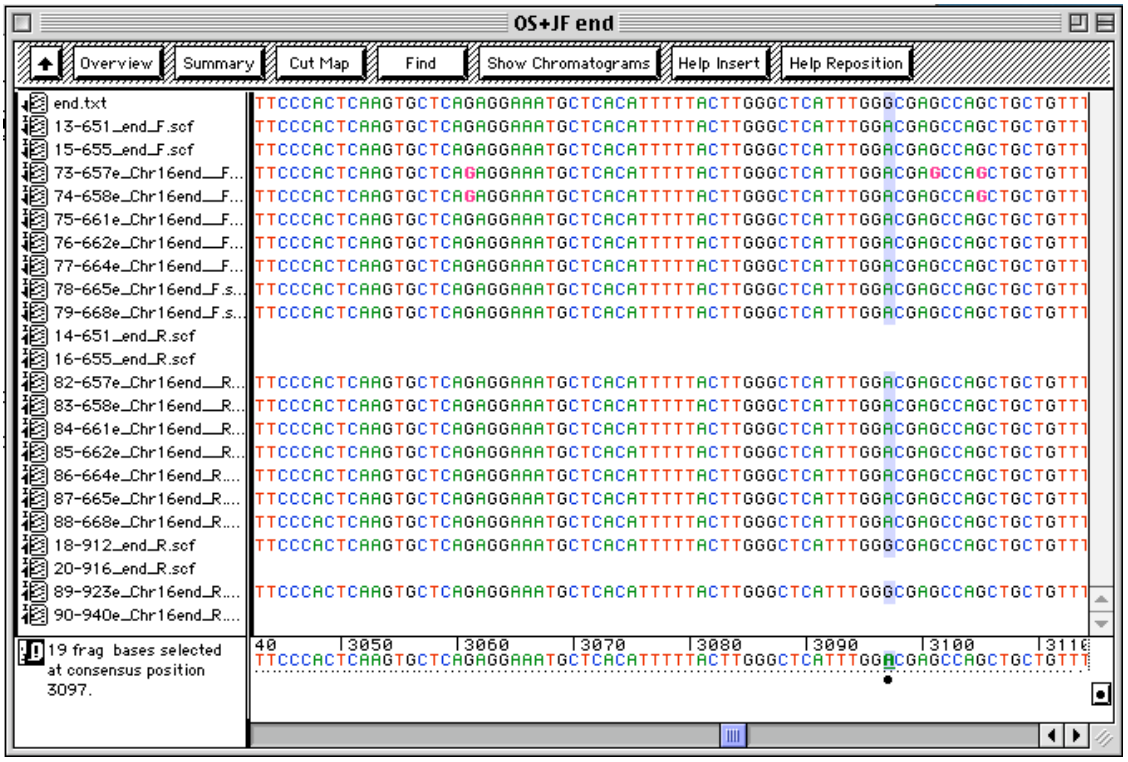
Base-pair position 472 in the start sequence.



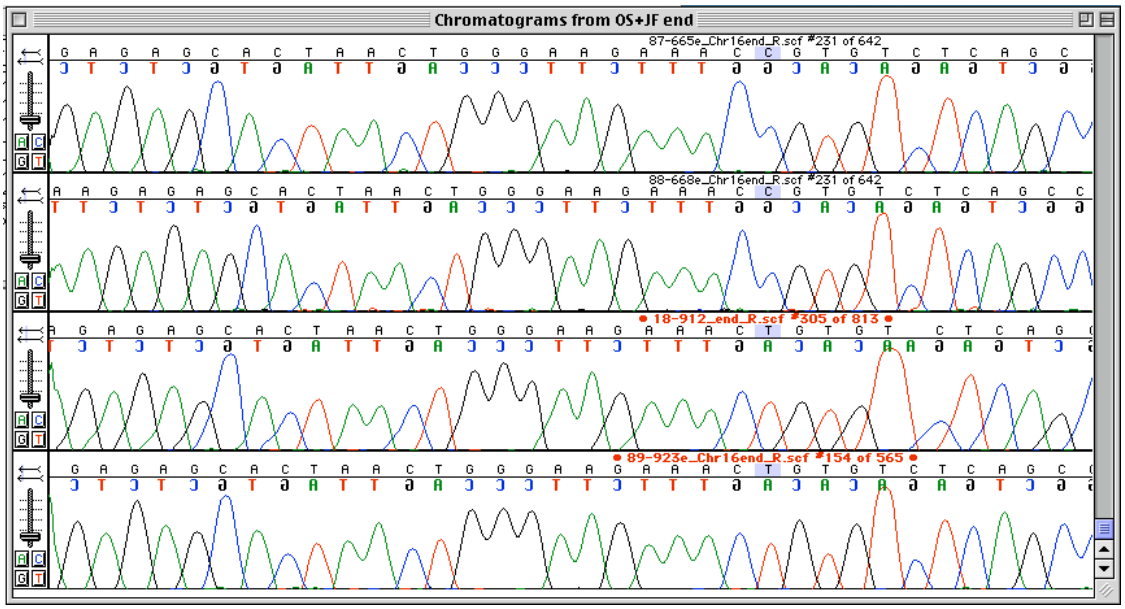
Base-pair position 496 in the start sequence.



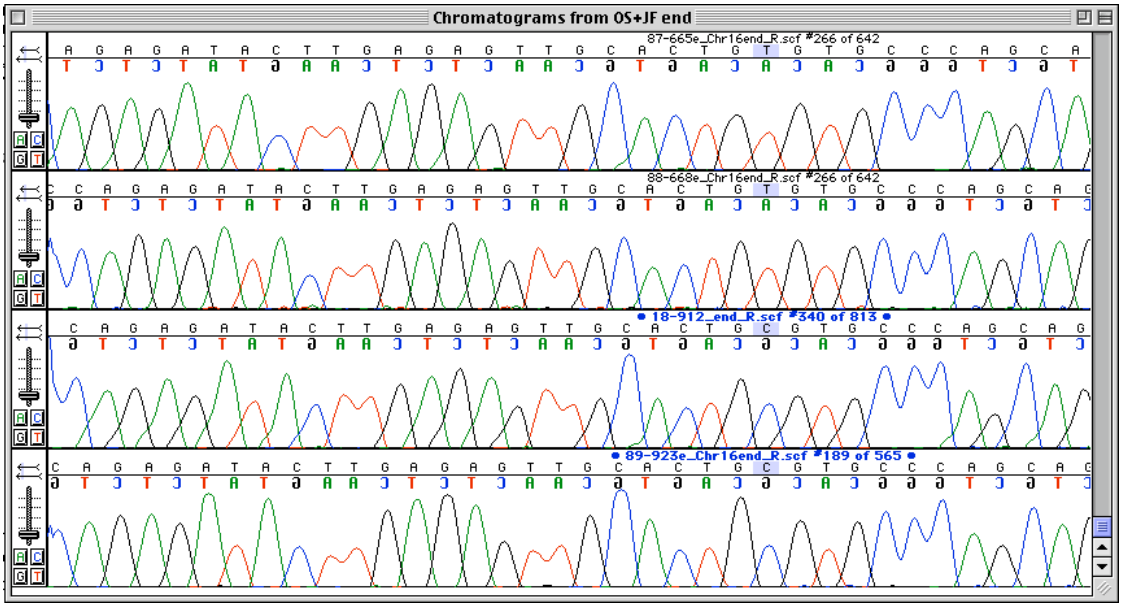
The two first of the informative SNPs in the end sequence. In base-pair position 2960 OS have C and JF T and in base-pair position 2995 OS have T and JF C.



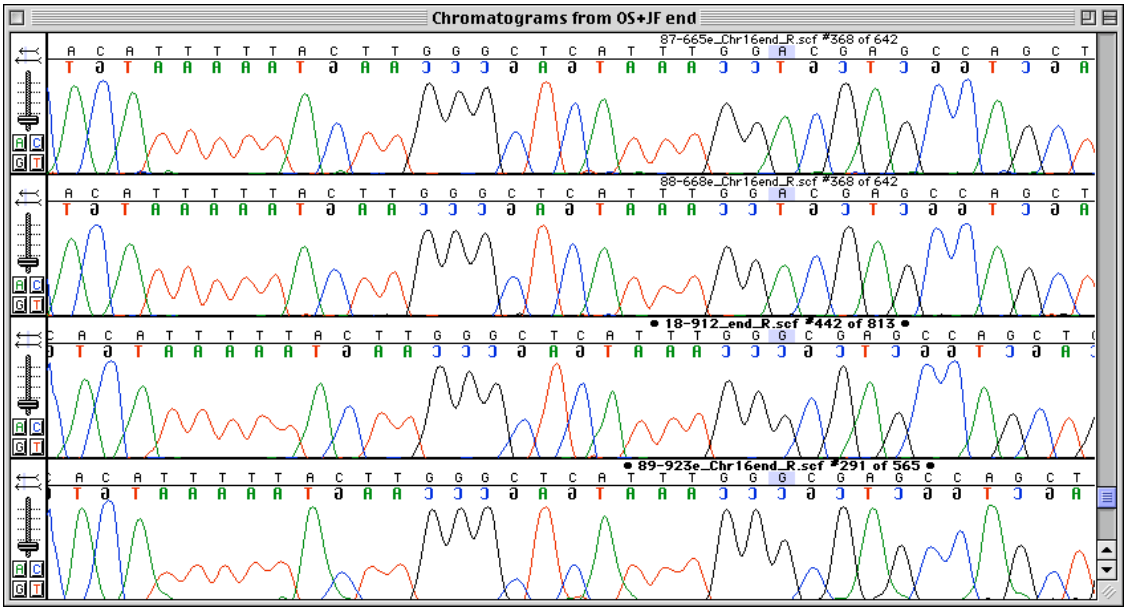
The third informative SNP found in the end sequence. In base-pair position 3097 OS have A and JF have G.



Base-pair position 2960 in the end sequence.

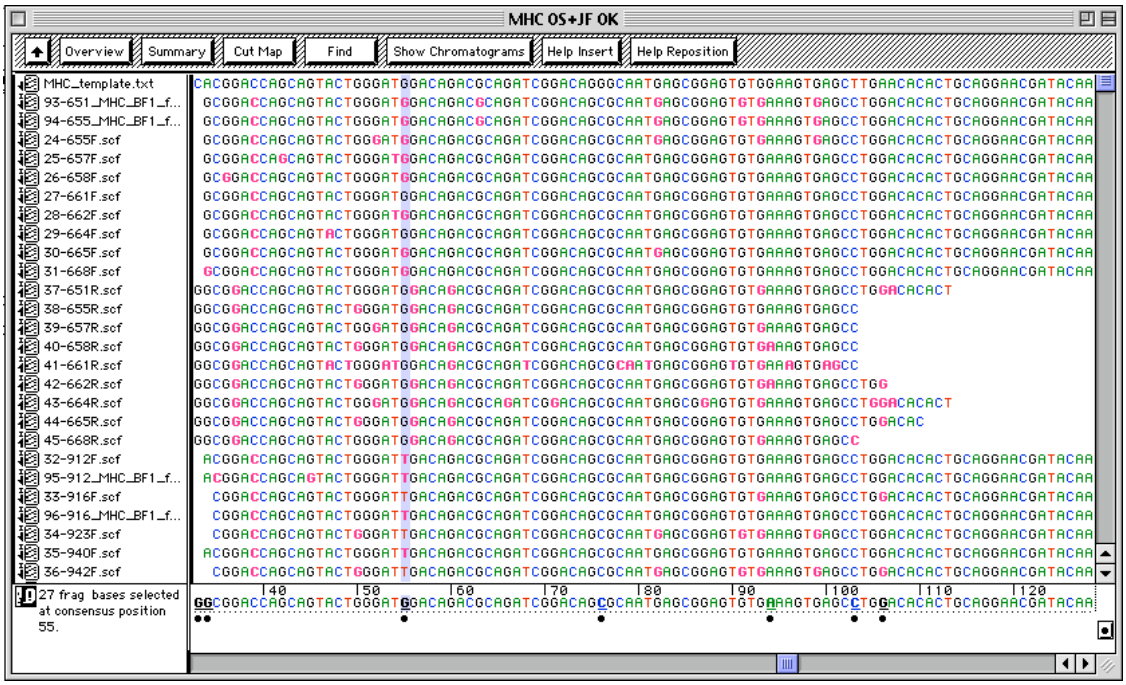


Base-pair position 2995 in the end sequence.

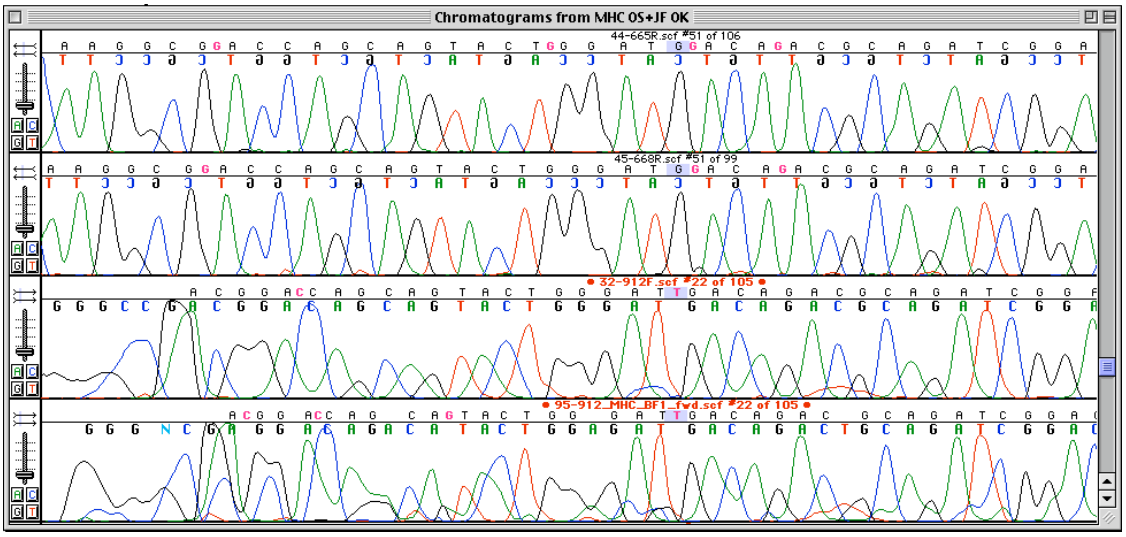


Base-pair position 3097 in the end sequence.

Appendix E, SNP in exon 2

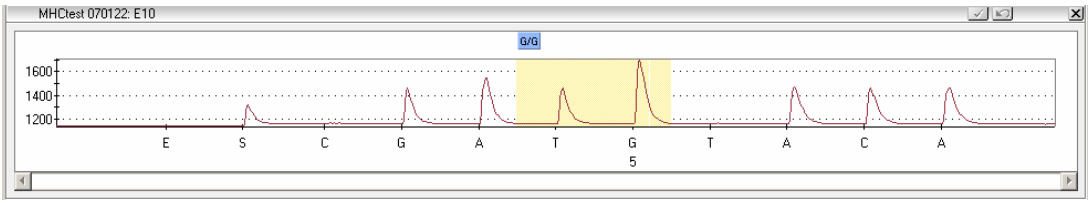


Informative SNP found in exon 2. In base-pair position 55 OS have G and JF T.



Base-pair position 55 in exon 2.

Appendix F, Pyrogram



Pyrogram from one of the individuals showing the G/G homozygote all individuals had.